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Stem cell-mediated regeneration of the infarcted heart

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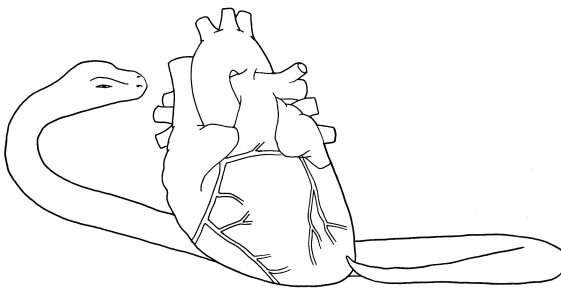
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Summary and future perspectives

S. Vandervelde



macrophages invaded the MI, peaked in numbers at day 7 and remained for at least four weeks post-MI. Moreover, we observed a rapid clearance of myocardial debris and the deposition of collagen for scar formation started within one week after MI. Adverse remodeling of the infarcted heart is already evident within the first week post-MI. Concluding from chapter 3 and 4, this process of post-MI inflammation was stronger and more rapid in both the cryo and reperfusion model compared to the ligation model. Remarkably, in both cryo and reperfusion there was also less severe adverse remodeling, indicating a supportive role for inflammation in remodeling.

Next, we set out to obtain molecular clues for the absence of spontaneous adequate cardiac regeneration after MI. In chapter 5 an overview of literature is given of the key signaling factors, i.e. cytokines and growth factors, involved in orchestrating the stem cell-driven repair processes of mobilization, homing, incorporation, survival, proliferation and differentiation. Infiltrated inflammatory cells are rich sources of cytokines and growth factors that play a role in the cardiac remodeling process following MI, but they may also dictate the behavior of stem cells in the cardiac repair process. Therefore, we investigated the expression of inflammatory factors relative to the expression of stem cell related factors and angiogenic factors in two clinically relevant models of MI. The results in chapter 6 indicate that early post-MI predominantly (pro-) inflammatory genes were strongly upregulated in the infarcted myocardium. In contrast, we found only marginal changes in the expression level of stem cell-related and angiogenic genes.

Thus, early after MI inflammation plays a dominant role and the cardiac microenvironment is crowded by infiltrated inflammatory cells and by expression of pro-inflammatory genes. An interesting cell in this respect is the CD14⁺ monocyte. CD14⁺ monocytes are the circulating progenitors of macrophages, but may also differentiate into endothelial progenitor cells (EPC) that are able to contribute to neovascularization [2,3]. In chapter 7 we stimulated human CD14⁺ monocytes to become either endothelial-like cells or macrophages. Our study revealed that the CD14⁺ monocyte is predestined towards a macrophage-like phenotype, even in the presence of endothelial-specific growth factors. Cultured CD14⁺ cells of stable angina pectoris patients had an even more pronounced macrophage-like phenotype compared to unstable angina pectoris patients. However, this type of cell is transcriptionally and metabolically active and is able to synthesize angiogenic factors, and may therefore be capable of augmenting neovascularization.

In conclusion, inflammation dominates the early post-MI murine cardiac microenvironment and interferes with optional stem cell-mediated cardiac regeneration. In addition, a supposed endothelial progenitor cell, the monocyte, is more inflammatory cell-like than expected, and can only be stimulated to contribute by paracrine effects to a part of cardiac regeneration, i.e. neovascularization. Therefore, for optimal stem cell-mediated regenerative therapy, the recipient post-MI cardiac environment needs to be transformed from an overwhelming adverse inflammatory state into a welcoming appropriate environment that support stem cells in their function to differentiate into different myocardial cells. However, the function of the immediate inflammatory response should not be underestimated, since it serves a role in prevention of acute adverse remodeling. Nevertheless, a proper balance between adverse inflammation and stem cell functioning is fundamental for stem cell-mediated

regenerative therapy.

Future perspectives

An ideal post-MI regenerative scenario would be, that only a few autologous pluripotent stem cells are activated or transplanted in the border area of the infarction. There, these cells need to proliferate, differentiate into various cell types, such as cardiomyocytes, endothelial cells, smooth muscle cells and other supporting cells, and form an electronically flawless continuum with the spared myocardium. Layer by layer the newly formed myocardium expands from the outer border of the infarct towards the middle to restore the infarcted myocardial tissue and thereby preserve cardiac function. To conduct this ideal scenario we have to meet with certain prerequisites.

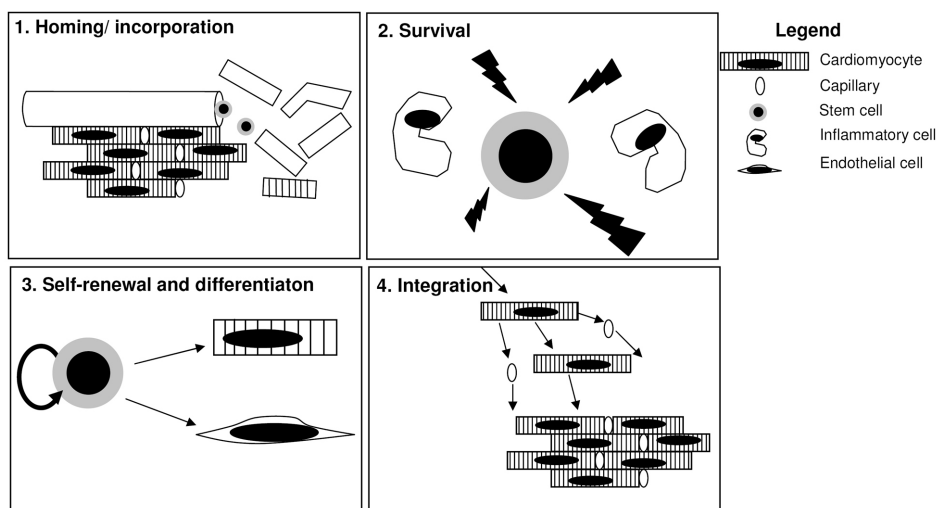


Fig. 1. Stem cell requirements for cardiac regeneration

Stem cell requirements

For stem cells to be able to function optimally, a few necessary steps need to be taken (Fig 1).

Firstly, stem cells need to be able to home to and incorporate in the infarcted myocardium. For this we need both attraction and retention signals. An exemplary signal for this is the chemokine SDF-1 and its receptor CXCR4 (chapter 5). SDF-1 is a very strong and dose dependant migratory attractant for a variety of cells that express the CXCR4 receptor, such as BMSCs, and by blocking of either SDF-1 or CXCR4 mobilization of stem cells from the bone marrow is inhibited [4,5]. We showed that the gene expression of SDF-1 did not increase post-MI in both investigated clinically relevant MI models (chapter 6). Microdissected regions revealed that within the infarcted region SDF-1 was even slightly downregulated, suggesting that without intervention the post-MI cardiac microenvironment lacks a strong attraction and retention signal. Moreover, progenitor cells overexpressing CXCR4 using a lentiviral gene transfer technique have increased chemotaxis towards lower doses of SDF-1 [6]. Also CXCR4 gene expression was not greatly increased post-MI, again

defeating chemotaxis of BMSCs.

Also, stem cells need to transmigrate through the endothelium into the damaged tissue, where there should be space for the stem cells to incorporate. We showed that the number of small vessels especially suitable for transmigration was dramatically decreased post-MI (chapter 3 and 4). Four days post-MI there was an increase in medium-sized and large vessels in all 3 models of MI, but not in small vessels. This may hamper transmigration of stem cells into the damaged tissue and therefore neovascularization of small vessels should be induced post-MI. Concerning the spatial distribution, the structural integrity of the scar tissue which is formed at the end of the wound healing process post-MI does not provide space for stem cells. We showed that collagen deposition started approximately 7 days post-MI in the ligation model, but started already at 4 days post-MI in the cryo and reperfusion model (chapter 3 and 4). However, tight scar formation was present as of 14 days post-MI (Fig 4 chapter 3, Fig 2 chapter 4) which leaves a time window for stem cell incorporation.

Secondly, stem cells need to survive in the infarcted area. Nutrients and oxygen should be available for the stem cells, in other words proper vascularization of the infarcted area is warranted. We showed that neovascularization as a part of the inflammatory response was evident after 4 days post-MI, but remarkably, as mentioned earlier, did not concern the small vessels, which are required for diffusion of nutrients and oxygen (chapter 3 and 4). Furthermore, stem cells should not be attacked by harmful factors that are often present in inflammatory environments, for example reactive oxygen species and proteases deriving from infiltrated neutrophils [7]. Especially in the first 4 days post-MI in the reperfusion and the cryo-injury MI model, until 7 days post-MI in the ligation model, neutrophils were present in large quantities in the infarcted area, which might jeopardize the survival of incorporated stem cells (chapter 3 and 4).

Thirdly, self-renewal, differentiation and maturation of stem cells into myocardial cell types should be stimulated. For regeneration of infarcted myocardium mostly cardiogenic and vasculogenic maturation of stem cells is needed. Signals that might be cardiogenic, as we discussed in chapter 5, were not greatly increased post-MI (chapter 6). Of the angiogenic genes investigated in chapter 6, only IL-8 was increased during the first two days post-MI. However, IL-8 is also involved in chemoattraction and activation of neutrophils (chapter 5). Thus, signals to suffice the third stem cell requirement need to be introduced for optimal stem cell-mediated cardiac regeneration. Furthermore, the maturation process should be carefully choreographed by either spared myocardium or potentially by the infiltrated stem cells themselves. In either way, a proper balance should be sought between post-MI inflammatory signals and regenerative signals. In this respect dosage, timing and location of signals and stem cells are of great influence. For example, G-CSF can mobilize stem cells from the bone marrow into the peripheral circulation [8], but G-CSF is also known to mature progenitor cells into neutrophils [9]. We showed that G-CSF was absent in the non-infarcted heart and was greatly induced immediately post-MI (chapter 6). Although this could be a signal for mobilization of stem cells from the bone marrow, this induction also coincided with high incidence of neutrophil infiltration in the infarcted area (chapter 4).

Last but not least, a structural continuum should be formed between the newly formed and residential spared myocardium. This step is especially complex concerning the delicate cardiac conduction system, which should provide a flawless smooth contraction signal to preserve optimal pump function. For example, in a clinical trial of engraftment of

Summary of thesis

Myocardial infarction (MI) and subsequent heart failure is an important cause of death in the Western world. Cardiac ischemia provokes an inflammatory response that leads to scar formation. The sustained presence of scar tissue after MI causes a gradual loss of cardiac function and may eventually lead to death. One way to intervene in this downward spiral, is to use stem cells to regenerate the infarcted myocardium. These stem cells ought to differentiate into cardiomyocytes, endothelial cells and supporting cells, which together functionally integrate with the spared myocardium and thus restore cardiac function. Stem cell-mediated cardiac regeneration depends on a welcoming appropriate recipient cardiac environment. However, after MI the strong inflammatory response may hamper optimal stem cell positioning and function. In this thesis, we investigated the role of inflammation in stem cell-mediated cardiac regeneration after myocardial infarction.

In chapter 2 we compared the functional effects of myocardial grafting of human skeletal myoblasts (SM) or CD133+ bone marrow-derived hematopoietic progenitors in nude rats after MI. Several studies indicate that engrafted skeletal myoblasts could increase cardiac function post-MI, but remain lineage restricted to their skeletal myoblastic phenotype [1]. However, bone marrow-derived stem cells are in general more plastic and therefore might regenerate the ischemic cardiac tissue more effectively. The results of our comparison indicated that both CD133+ progenitors and skeletal myoblasts grafted intramyocardially 10 days post MI similarly improved the preservation of LV function compared to non-grafted controls. Although the SM were administered in high doses (5×10^6 grafted cells), we detected only a few in the myocardium by co-expression of human nuclear and skeletal muscle specific markers. However, we were not able to detect the grafted CD133+ cells (5×10^5 grafted cells) by various immunohistological stainings. The reasons for the CD133+ graft loss remained unclear. We speculated that the post-MI inflammatory process negatively influences survival of bone marrow-derived stem cell grafts.

Therefore, we investigated the post-infarction cardiac environment in which (engrafted) stem cells need to function. In chapter 3 and chapter 4 we explored the inflammatory reaction following MI in different murine models of MI. In chapter 3 we compared a cryo MI model, in which cryoinjury is applied to the epicardial site of the LV ('cryoinjury'), with a MI model of permanent ligation of a coronary artery branch ('ligation'). Ligation resulted in marked adverse remodeling and decreased systolic cardiac function compared to cryoinjury. In addition, cryoinjury of the LV wall resulted in an increased inflammatory cell infiltration, accelerated scar formation and increased neovascularization compared to ligation.

In chapter 4 we compared the model of permanent ligation with a model of transient ligation of the similar branch of the coronary artery followed by reperfusion ('reperfusion'). Ligation resulted in a higher mortality rate and in increased adverse remodeling compared to reperfusion. In addition, after reperfusion, infarcts had a higher inflammatory cell influx, had a faster clearance of cardiomyocyte remnants and showed earlier deposition of collagen. Moreover, reperfusion resulted in an increased number of new vessels compared to ligation.

Overall, all three models of MI resulted in an early inflammatory response characterized by a major influx of inflammatory cells. The influx of neutrophils is mainly in the first two days post-MI, after which they rapidly disappeared. Following the neutrophil influx

skeletal myoblasts ventricular tachycardia developed in several cases despite histological confirmation of engraftment [10]. Although arrhythmias are not observed in clinical bone-marrow stem cells studies so far [11], abundant differentiation into cardiomyocytes and formation of a optimal conduction system in continuum with the spared myocardium has not been reported yet.

Post-MI stem cell-mediated therapy

Several experimental methods are developed for stem-cell mediated cardiac regenerative therapy. The first and most widely used method is stem cell transplantation. Exogenous (autologous) stem cells derived from the bone marrow or the peripheral blood are transplanted in or round the infarcted area, either catheter based via the coronary arteries or directly by injection during open-heart surgery. In chapter 2 we used the transplantation method to compare the effectiveness of skeletal myoblasts with CD133 progenitor cells. Meanwhile, the engraftment method has also been used in clinical experiments [12,13]. These studies showed that clinical stem cell engraftment is feasible, safe, and that it improves cardiac functioning. Later placebo double blind controlled studies showed similar results [14], although clinical improvement was less great than initially expected and some clinical studies show no significant functional effects [15]. We did observe functional improvement one month after intracardiac transplantation of high amounts of both human skeletal myoblasts and CD133 in a ligation MI model in nude rats , but histologically we detected only a few of the grafted myoblasts and no CD133 cells. Also others observed transplantation yielded low sufficiency [16]. Possible reasons for graft loss are seeding insufficiency, for example because of lack of vascularization early after infarction or because transplanted cells leak into the systemic circulation. Another reason for graft loss can be impeded graft survival, for example because harmful factors arise from the post-MI inflammation. For optimization of the transplantation method, future investigations need to focus on finding 1) the optimal dose of stem cells, 2) the optimal timing of transplantation to assure survival and to meet spatial requirements, and 3) the optimal localization of transplantation of stem cells. Of course, 4) optimizing the cardiac microenvironment that is hosting the transplanted stem cells should also be a focus in future investigations and will be further discussed below.

The second method is based on enhancement of mobilization of stem cells from the bone marrow into the peripheral blood to increase availability of stem cells for cardiac regeneration. Enhancement of mobilisation can be obtained by intravenous injection of for example G-CSF [17-19]. First clinical trials indicate G-CSF administration post-MI is safe [20,21], and may improve LV dimensions and cardiac function [22]. However, along with these results it appeared that G-CSF-based therapy seems to attenuate remodeling by undefined paracrine effects, so the improvement did not arise from stem cell-mediated cardiac regeneration. However, recently no sustained improvement in left ventricular dimensions and cardiac function could be observed at all in two clinical double-blind placebo controlled G-CSF trials [23,24]. We observed a high expression of G-CSF immediately post-MI in mice, which was similar in the reperfusion model and ligation model, although reperfusion resulted in less severe adverse remodeling (chapter 6). This might indicate that G-CSF on its own does not govern attenuation of remodeling. Nevertheless, mobilization by other cytokines or growth factors need to be further explored and may still facilitate cardiac regeneration.

The third method is based on optimization of the local recipient infarcted microenvironment. This method aims at the four stem cell requirements mentioned above. An example of optimizing homing and incorporation is engraftment of stably transfected cardiac fibroblasts overexpressing SDF-1 [25]. This resulted in higher numbers of BMSCs in the heart and in augmented vascularization and cardiac function. Another interesting example of optimizing the local cardiac environment for stem cells is the administration of small growth factors attached to a biomaterial carrier to provide sustained delivery within the infarcted area of a regenerative-prone factor [26]. In this specific case insulin-like growth factor-1 (IGF-1), a factor that, among other things, can promote cardiogenesis (chapter 5), was delivered by biotinylated nanofibers, which combined with cell therapy, resulted in improved cardiac function post-MI [26]. However, these experiments are only conducted in preclinical settings so far.

Post-MI cardiac microenvironment

To be able to optimize the cardiac niche for stem cell-mediated regenerative therapy we have to intervene in the sequence of events of the inflammatory response that starts immediately post-MI, which preludes the adverse remodeling of the infarcted heart (Fig 2). One of the key question in this respect is whether suppression of the inflammatory response is sufficient to reach the goal of optimizing the local environment for stem cells?

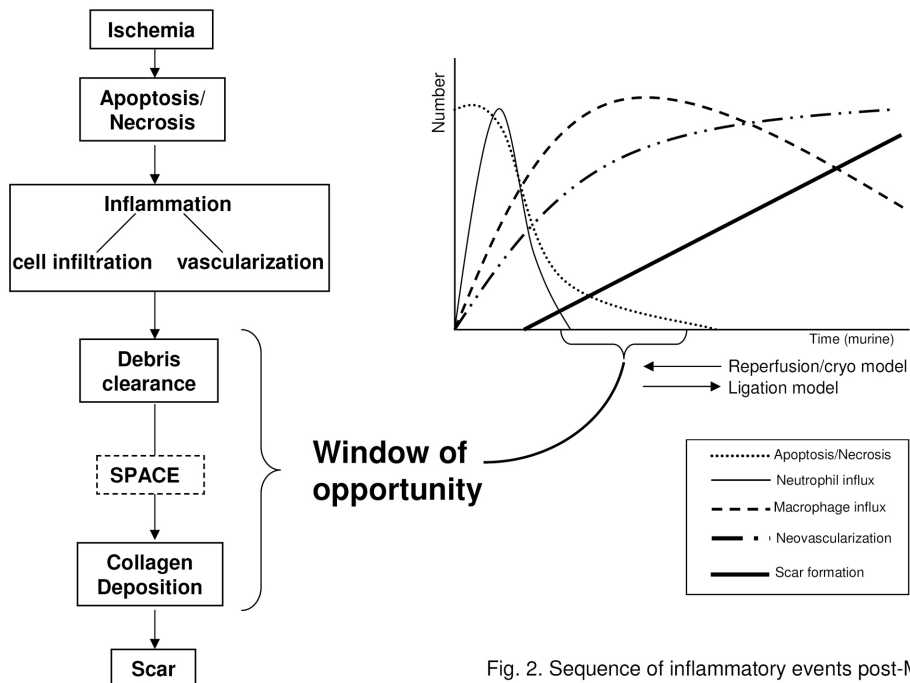


Fig. 2. Sequence of inflammatory events post-MI.

Several (clinical) investigations attempted to reduce the inflammatory response post-MI. Administration of corticosteroids to patients suffering from acute MI resulted in increased infarct sizes and higher incidence of ventricular arrhythmias, despite initial success in animal models [27,28]. Investigations using free radical scavengers in patients with acute MI undergoing thrombolysis or balloon angioplasty did not result in significant improvement of cardiac function [29, 30]. In our laboratory, an experiment in which circulating monocytes were depleted and thereby post-MI macrophage accumulation in the infarcted area was prevented, resulted in increased adverse cardiac remodeling of the cryo-injured heart (M. van Amerongen et al., unpublished data). Concluding from our own experiments (chapter 3 and 4), the accelerated and augmented inflammatory response in both the cryo MI and the reperfusion MI model compared to the ligation model was concomitant with less severe adverse remodeling.

Therefore, we cannot deny the importance of the inflammatory response post-MI, which is a prerequisite for the wound healing response resulting in scar formation. However, sustained inflammation can also extend myocardial injury and interfere with optional stem cell functioning by providing non-regenerative signals in the cardiac microenvironment. The challenge therefore lays in timing of intervention to switch from the inflammatory hostile environment into a calm environment that meets the four stem cell requirements mentioned earlier. Future investigation should reach for the time window between the necessary clearance of cell debris by macrophages and the start of tight scar formation (Fig 2). In that short time window, there is space to incorporate, factors that could endanger the survival of stem cells mostly subsided and neovascularization has started, indicating the first two stem cell requirements are almost fulfilled. However, this small time window is framed by the necessity to maintain the strength of the ventricular wall to prevent cardiac rupture.

Intervention that is needed to accomplish the other stem cell requirements are 1) implementation in the infarcted area of signals to attract, activate and retain stem cells, and 2) signals to mature stem cells into cells of myocardial lineages, i.e. mainly cardiomyocytes and endothelial cells. An example of a future experiment that pursues manufacturing of the ideal cardiac niche for stem cell regeneration is the intracardiac implementation of a combination of factors such as SDF-1, IGF-1 and VEGF (chapter 5) so that stem cells are attracted to the MI (SDF-1) and receive maturation signals to become both cardiomyocytes (IGF-1) and endothelial cells (VEGF). A way to maintain these small factors together in place and to achieve sustained delivery is by attaching them to flexible biomaterial that serves as carrier and that can be engrafted in the MI [31].

Optimization by adaptation of macrophage behavior

Another possibility to optimize the local environment focuses on a specific player of the inflammatory response post-MI: the macrophage. Only recently, macrophages are under thorough investigation for their ability to adapt their phenotype to their local environment [32]. Our *in vitro* data showed that monocytes (progenitor of macrophages) might be able to contribute to neovascularization by synthesizing and secretion of VEGF-a without differentiating into endothelial cells (chapter 7). This quality of inducing paracrine effects may potentially be extended if macrophages are stimulated to synthesize and secrete regenerative-prone proteins. Moreover, if macrophages can also be suppressed to secrete pro-inflammatory and pro-collagenic factors, this cell could serve most of the

regeneration requirements. However, the potency of macrophages is only recently recognized and future investigation need to proof that macrophages are not only vigorous, but that they can also be modulated. Moreover, we showed that monocytes of patients with stable angina pectoris had a more pronounced macrophage-like phenotype (chapter 7), which implies that differentiation potential and phenotype are influenced by disease and perhaps also by age, which makes the assignment to modulate macrophages to optimize the infarcted microenvironment even more challenging.

Finally, in this thesis on the role of inflammation in stem cell-mediated cardiac regeneration, we showed that after myocardial infarction inflammation dominated the infarcted microenvironment, which presumably hampers stem cells to regenerate. Therefore, we suggest to aim future investigation at creating a balance between adverse inflammatory signals and regenerative-prone signals. Hence, fine tuning of the inflammatory response is elementary for post-MI regenerative therapy.

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